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Comparative studies of two acid β-galactosidases from rabbit and bovine kidney

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Comparative studies of two acid β-galactosidases from rabbit and bovine kidney. Comparative studies of two acid β-galactosidases from rabbit and bovine kidney have been made. The enzyme from rabbit (enzyme I) was purified 450-fold, and the activity of the bovine enzyme (enzyme II) was enriched 250-fold using conventional purification methods. Both purified enzymes were characterized and their properties were compared. Enzyme I showed a lower optimal temperature and was less pH stable. Enzyme II appeared to be homogeneous in charge, in contrast to the heterogeneity observed for enzyme I. Studies on their specificity using natural substrates showed that enzyme I hydrolyzed G_{M1}, asialofetuin and lactose. However, enzyme II was only able to cleave galactose from the disaccharide. Some of the carbohydrates tested acted as activators for enzyme II, suggesting a mechanism of transglycosilation. Using lactose as substrate we confirmed the ability of enzyme II to transfer galactose residues to D-maltose and N-acetylgalactosamine.

Acid β-D-galactosidase (E.C. 3.2.1.23) is known to be involved in the catabolism of carbohydrates [1], glycolipids and glycoproteins [2]. This enzyme has been the subject of intense investigation both because there is interest in exploring its chemical, physical and enzymatic properties [3–5] and because an inborn deficiency of acid β-galactosidase is the primary enzyme defect in the human lysosomal storage disorder, G_{M1} gangliosidosis [6].

Mammalian β-galactosidase has been characterized in many tissues, but the studies on its specificity are still incomplete and the biological role of the enzyme is unclear. At present, some of the uncertainties are probably due to the multiplicity of the enzyme and the use of different synthetic and natural substrates. A comparison of β-galactosidase from different mammalian sources may help in the understanding of the metabolic significance of this enzyme.

Previous studies in our laboratory showed a close physical and chemical relationship between rabbit acid β-galactosidase from kidney [7] and spleen [8], indicating that the different location does not cause major differences in their properties, as suggested by Cheetham [9]. In the present work, two β-galactosidases purified from the same tissue of different sources, rabbit and bovine kidney, were compared and some studies on their specificity using natural substrates were carried out.

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Methods

Bovine and rabbit kidneys were obtained fresh from normal female and male animals, immediately after death, and stored at −20°C until used.

Enzyme assays

β-Galactosidase activity was determined as previously described [7]. Unless otherwise specified, incubation mixtures contained 0.1 M Mc'Ilvaine buffer (pH 4.0 for enzyme I and pH 4.5 for enzyme II), 100 μl of enzyme solution and 3 mM *p*-nitrophenyl-galactoside in a final volume of 1 ml. One unit of enzyme (U) was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions.

The ability of β-galactosidase to release galactose residues from natural substrates was investigated using asialofetuin (12 mg/ml), lactose (12 mg/ml) and G_{M1} ganglioside (3 mg/ml). Incubation mixtures contained 0.5 ml of enzyme solution and 1 ml of substrate previously solubilized in Mc'Ilvaine buffer. When using G_{M1} ganglioside, 0.2% (vol/vol) Triton X-100 and 0.1% (wt/vol) sodium taurocholate were also added to ensure the substrate solubility. After incubation at 37°C for 96 hours the reaction was stopped by freezing. Controls, in the absence of the enzyme, were also included to determine any non-enzymatic hydrolysis. The cleavage of each substrate was calculated by determination of free galactose [10] and free glucose [11].

Protein determination

Protein concentration was determined by the method of Lowry et al [12] using bovine serum albumin as the standard.

Enzyme purification

All purification steps were carried out at 4°C. The rabbit enzyme was purified using affinity chromatography on concanavalin A-sepharose and *p*-aminophenyl-thio-β-D-galactopyranoside agarose (PAPS-Agarose) as previously described [7].

Bovine kidneys were minced and homogeneized in distilled water (3 ml/g) in an "Omnimixer"(4 periods at 6,000 rpm). The crude homogenate was centrifuged for 20 min at 20,000 × g. The supernatant was again centrifuged for 60 minutes at 100,000 × g and the pellet was discarded. The supernatant was then

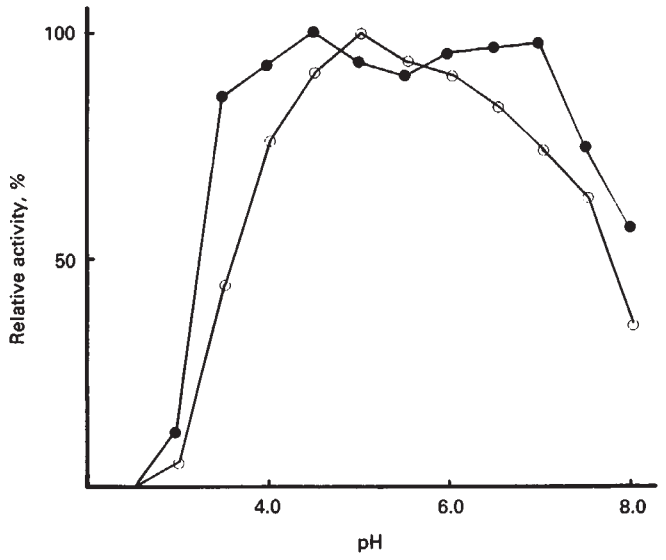

Fig. 1. *pH stability of rabbit (○—○) and bovine (●—●) acid β-galactosidases.* Assays were conducted as described in **Methods.**

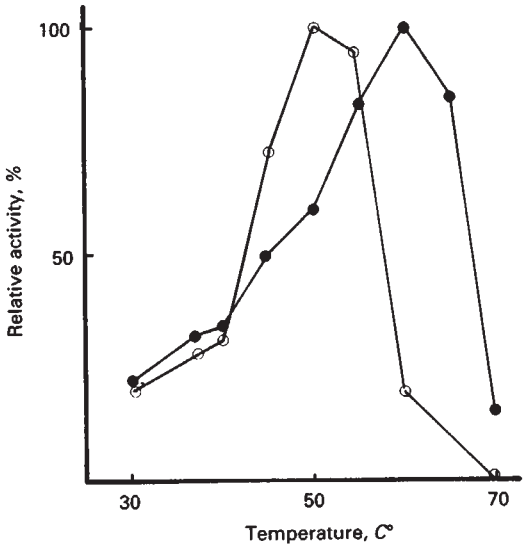

Fig. 2. *Effect of temperature on acid β-galactosidase activity.* Symbols are: enzyme I (○—○); enzyme II (●—●). Experimental details are given in the text.

applied to a concanavalin A-sepharose column (0.5 × 15 cm) equilibrated with 10 mM phosphate buffer, pH 6.5. After washing the unadsorbed proteins, the acid β-galactosidase was eluted with α-methyl-mannoside and 1 M KCl in the phosphate buffer. Concanavalin A-sepharose purified β-galactosidase was dialyzed against distilled water for 24 hours and applied to a DEAE-cellulose column (2 × 20 cm) equilibrated with 10 mM phosphate buffer, pH 6.5. The same solution was used to elute the unadsorbed proteins and then a linear gradient of KCl was used to release the bound proteins.

Effect of pH and temperature

Optimal pH was determined by replacing the buffer in the standard assay mixture for Mc'Ilvaine and phosphate buffers at several pH values, ranging from 2.6 to 5.5 and 5.5 to 8.0, respectively. Enzyme pH stability was tested by previous incubation of the enzyme at 37°C for one hour in buffer solution at different pH values. The remaining activity was measured as described above. The effect of temperature was tested incubating the enzyme for 20 minutes at different temperatures in the range from 30°C to 60°C. Thermal stability of the enzyme was determined by measuring the remaining activity after preincubation in 0.1 M Mc'Ilvaine buffer in a temperature range from 40°C to 60°C.

Isoelectric focusing

The electrofocusing of the enzymes was carried out on a LKB 8,100 column (110 ml) according to Vesterberg [13], using ampholytes (1% vol/vol) within a pH range from pH 3.0 to pH 10 in a linear sucrose gradient. Electrofocusing was allowed to proceed for 48 hours at 500 V. Two milliliter fractions were collected and analyzed for pH and enzyme activity.

Effect of some carbohydrates

β-Galactosidase activity was estimated in the presence of several carbohydrates added to the standard reaction mixture. In every case the final concentration of the effectors was 100 mM. Controls without the carbohydrates were used.

Galactosyltransferase assay

The galactosyltransferase activity of the bovine enzyme was studied using lactose as the galactose donor and maltose and *N*-acetylgalactosamine as potential acceptors. The concentration of the carbohydrates added to the standard reaction mixture was 210 mM. Free galactose and glucose were determined as mentioned before. The enzymatic assay was carried out at pH 4.5. Control tubes lacking the acceptors were also used.

Results

Purification of acid β-galactosidase from rabbit and bovine kidney

The purification of rabbit kidney acid β-galactosidase (enzyme I) was achieved in a previous work [7] using concanavalin A-sepharose and PAPS-agarose. The enzyme was purified 450-fold with a yield of 11% and it exhibited a specific activity of 2.7 U/mg.

The β-galactosidase from bovine kidney (enzyme II) was purified using concanavalin A-sepharose and DEAE-cellulose. The activity was enriched about 250-fold and the overall recovery was 9% of the activity in the homogenate.

Both purified enzymes showed a single band in polyacrylamide gel electrophoresis and other glycosidase contaminants were absent (data not shown).

Optimal pH and pH stability

The optimal pH of enzyme I was 4.0 while enzyme II showed the maximal activity at pH 4.5. The preincubation at several pH values showed a different stability of both enzymes (Fig. 1). Enzyme I retained almost a 100% of its activity between pH values of 4.5 and 6.0, while enzyme II showed maximal activity in a wider pH range, between pH 4.0 and 7.0.

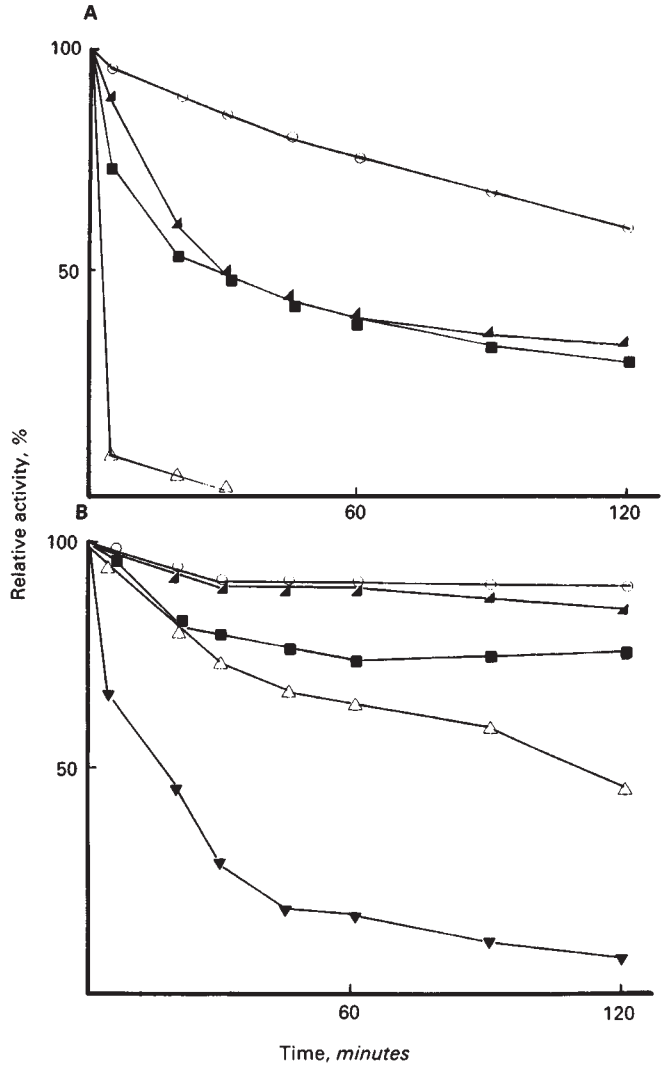

Fig. 3. *Enzyme stability at different temperatures: A. enzyme I; B. enzyme II.* Symbols for temperatures are as follows: 40°C, (○——○); 45°C, (◢——◢); 50°C, (■——■); 55°C, (△——△) and 60°C, (▼——▼).

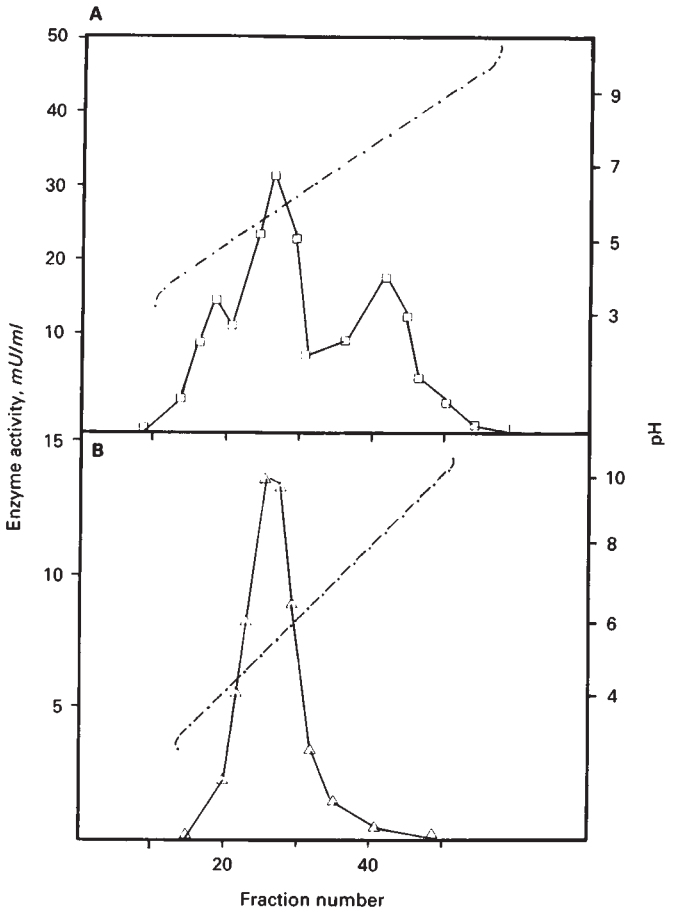

Fig. 4. *Electrofocusing profile of β-galactosidase activity in rabbit kidney* (**A**) *and bovine kidney* (**B**). Symbol is: (-· ·· -) pH of each fraction.

Optimal temperature and thermostability

As shown in Figure 2, the in vitro optimal temperature is 50°C for enzyme I and 60°C for enzyme II. Again, a different behavior was observed when both enzymes were preincubated at several temperatures. Enzyme I lost more than 50% of its activity when the preincubation was carried out above 40°C, even in short periods of time (Fig. 3A). In contrast, enzyme II retained 70% of activity after two hours of preincubation at 50°C, and was still active at 60°C after the same period of time (Fig. 3B).

Isoelectric point

As observed in Figure 4A, after isoelectric focusing enzyme I showed three peaks with pI values of 4.8, 6.0 and 7.3 [7]. In contrast, enzyme II ran as a single peak with an isoelectric point of 5.2 (Fig. 4B).

Table 1. Effect of some carbohydrates on enzyme I and II

Effector 100 mM	Relative activity % Enzyme I	Enzyme II
	100	100
D-Glucose	100	151
D-Galactose	102	6
D-Mannose	96	114
D-Fucose	87	137
D-Maltose	83	154
γ-D-Galactonolactone	5	11
γ-D-Gluconolactone	90	49
N-Acetylgalactosamine	92	166

Effect of some carbohydrates

The ability of several carbohydrates to affect the activity of both enzymes was tested and the results are shown in Table 1. γ-D-Galactonolactone was a strong inhibitor of both enzymes, while D-galactose and γ-D-gluconolactone only inhibited the activity of enzyme II. The carbohydrates D-glucose, D-maltose and *N*-acetylgalactosamine did not affect enzyme I but were very strong activators of enzyme II.

Table 2. Activity of enzymes I and II towards natural substrates

	Lactose	Asialofetuin	G_{M1}-ganglioside
	nmol gal/min/mg		
Enzyme I	4.24	0.39	1.5×10^{-4}
Enzyme II	134	0.0	0.0

Enzymatic hydrolysis of natural substrates

Using asialofetuin, G_{M1} ganglioside and lactose as natural substrates the enzymes displayed a different behavior (Table 2). Enzyme II was not able to hydrolyze either the glycolipid or the protein. In contrast, enzyme I cleaved the G_{M1} ganglioside at a rate of 1.5×10^{-4} nmol of galactose/min/mg. The resulting G_{M2} ganglioside was observed by thin layer chromatography (data not shown). Enzyme I also cleaved asialofetuin and the amount of galactose liberated was 0.39 nmol/min/mg. The disaccharide lactose was hydrolyzed by both enzymes, enzyme II being much more active (134 nmol/min/mg) than enzyme I (4.24 nmol of galactose/min/mg).

Galactosyltransferase activity

D-maltose and *N*-acetylgalactosamine were used as possible acceptors of the galactose cleaved during the hydrolysis of lactose. The results of these experiments are summarized in Table 3. As can be expected, in the absence of acceptors (control) the hydrolysis of lactose yielded equimolar amounts of galactose and glucose. In contrast, when *N*-acetylgalactosamine or maltose were present in the reaction mixture the concentration of free galactose was drastically reduced compared to the free glucose. Moreover, the hydrolysis of lactose (evaluated as amount of glucose liberated) was clearly activated, as occurred when using the synthetic substrate (Table 1).

Discussion

Kidney acid β-galactosidases from rabbit (enzyme I) and from calf (enzyme II) have been purified, characterized and their substrate specificity has been determined. Both enzymes were purified by conventional enzyme fractionation procedures. Purification of the rabbit enzyme was achieved in a previous work [7] and included affinity chromatography on concanavalin A-sepharose and PAPS-agarose. Enzyme II was purified using concanavalin A-sepharose and DEAE-cellulose chromatographies.

Enzymes I and II showed a similar rate of purification (450- and 250-fold, respectively) and a similar recovery (11% and 9%); they also showed a single band in polyacrylamide gel electrophoresis and were free of glycosidase contaminants. The data suggest that the purification achieved was similar for both enzymes, thus allowing for the comparative studies.

After purification, the enzymes were characterized and, even though both had a similar optimal pH (around 4.0), they differed in most of the properties studied. When the enzymes were preincubated at several pH values (Fig. 1) and temperatures (Fig. 3), enzyme II appeared to be more stable compared to enzyme I. The in vitro optimal temperature was 50°C for enzyme I and 60°C for enzyme II (Fig. 2).

Heterogeneity in charge has been described for acid β-galactosidase in different tissues [14–16]. After isoelectric focusing, enzyme I showed three pI values, as can be observed in Figure 4A [7]. In contrast, when enzyme II was electrofocused, only one peak of activity at pH 5.2 was seen (Fig. 4B).

Table 3. Transferasic activity of enzyme II using lactose as substrate and D-maltose and *N*-acetylgalactosamine as acceptors

Acceptor	Free galactose after hydrolysis	Free glucose after hydrolysis	Activation	Transferred galactose
	nmol/mg/min		*%*	
—	134	151	100	—
D-Maltose	99	943	624	90
N-Acetylgalactosamine	110	202	134	46

To study the specificity towards natural substrates, we used lactose, asialofetuin and G_{M1} ganglioside (Table 2). Enzyme I hydrolyzed the glycolipid and showed substantial activity against asialofetuin, thus suggesting a role for the enzyme in glycolipid and glycoprotein degradation. The disaccharide lactose was hydrolyzed by both enzymes, although enzyme I displayed a very low rate of activity (Table 2).

Both enzymes were affected by carbohydrates in a different way (Table 1). The major difference was observed when D-glucose, D-maltose and *N*-acetylgalactosamine were included in the reaction mixtures. All three compounds were very strong activators of enzyme II while enzyme I was not affected. The activation of enzyme II by some of the carbohydrates tested could be explained by a mechanism of transglycosylation [17] since an increase in the acceptor concentration resulted in an enhanced formation of the transfer product [18].

To test the ability of enzyme II in the transfer of galactose residues to D-maltose and *N*-acetylgalactosamine we included these compounds in the hydrolysis reaction mixture using lactose as substrate. The results were conclusive (Table 3); the enzymatic hydrolysis of lactose would be expected to yield equimolecular amounts of galactose and glucose as occurred in controls. However, when maltose or *N*-acetylgalactosamine were added, the release of galactose from lactose was apparently reduced. On the contrary, the amount of free glucose was higher compared to the control. These results can only be explained considering that, in the presence of the two mentioned carbohydrates, increased hydrolysis occurred (as observed when using *p*-nitrophenyl-galactopyranoside as substrate), and that a portion of the galactosyl residues were transferred to the acceptors. The acidic pH used for these assays, and the high purity of enzyme II ensured that the galactosyl transferase activity detected was really due to the enzyme rather than any possible contaminant.

Preliminary studies to investigate the ability of enzyme I to transfer galactose residues to some acceptors were not successful, which is consistent with the lack of activation by carbohydrates.

The propensity of many glycosidases to carry out glycosyl transfer reactions is well documented [18–23]. One could infer a possible role for the transferase activity in sugar transport, but the complete physiological importance of this β-galactosidase-catalized transglycosilation reaction in mammals has yet to be fully investigated.

Observations made in our laboratory studying the rabbit

enzyme from spleen [8] and kidney [7] agreed with those of Cheetham [9], who found that the properties of rabbit liver β-galactosidase corresponded broadly with those of the brain enzyme, indicating that the different environment and metabolic role of the liver and brain enzymes does not cause major differences in the number and properties of β-galactosidase isoenzymes. In contrast, the present studies indicate that two mammalian β-galactosidases from rabbit and bovine kidney differ in most of the studied properties, suggesting a different role for this enzyme in the same tissue from different species.

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